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The cold shock protein (Csp) family comprises small, highly conserved proteins that bind nucleic acids to modulate various bacterial gene expressions. In addition to cold adaptation functions, this group of proteins is thought to facilitate various cellular processes to promote normal growth and stress adaptation responses. Three proteins making up the Listeria monocytogenes Csp family (CspA, CspB, and CspD) promote both cold and osmotic stress adaptation functions in this bacterium. The contribution of these three Csps in the host cell invasion processes of L. monocytogenes was investigated based on human Caco-2 and murine macrophage in vitro cell infection models. The DeltacspB, DeltacspD, DeltacspAB, DeltacspAD, DeltacspBD, and DeltacspABD strains were all significantly impaired in Caco-2 cell invasion compared with the wild-type strain, whereas in the murine macrophage infection assay only, the double (DeltacspBD) and triple (DeltacspABD) csp mutants were also significantly impaired in cell invasion compared with the wild-type strain. The DeltacspBD and DeltacspABD mutants displayed the most severely impaired invasion phenotypes. The invasion ability of these two mutant strains was also further analyzed using cold-stress-exposed organisms. In both cell infection models a significant reduction in invasiveness was observed after cold stress exposure of Listeria organisms. The negative impact of cold stress on subsequent cell invasion ability was, however, more severe in cold-sensitive csp mutants (DeltacspBD and DeltacspABD) compared with the wild type. The impaired macrophage invasion and intracellular growth of DeltacspBD and DeltacspABD also led us to examine oxidative stress resistance capacity in these two mutant strains. Both strains also displayed higher oxidative stress sensitivity relative to the wild-type strain. Our data indicate that besides cold and osmotic stress adaptation roles, Csp family proteins also promote efficient host cell invasion and oxidative stress adaptation processes in L. monocytogenes.

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Reduced Host Cell Invasiveness and Oxidative Stress Tolerance in Double and Triple *csp* Gene Family Deletion Mutants of *Listeria monocytogenes*

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Abstract

The cold shock protein (Csp) family comprises small, highly conserved proteins that bind nucleic acids to modulate various bacterial gene expressions. In addition to cold adaptation functions, this group of proteins is thought to facilitate various cellular processes to promote normal growth and stress adaptation responses. Three proteins making up the *Listeria monocytogenes* Csp family (CspA, CspB, and CspD) promote both cold and osmotic stress adaptation functions in this bacterium. The contribution of these three Csps in the host cell invasion processes of *L. monocytogenes* was investigated based on human Caco-2 and murine macrophage *in vitro* cell infection models. The Δ*cspB*, Δ*cspD*, Δ*cspAB*, Δ*cspAD*, Δ*cspBD*, and Δ*cspABD* strains were all significantly impaired in Caco-2 cell invasion compared with the wild-type strain, whereas in the murine macrophage infection assay only, the double (Δ*cspBD*) and triple (Δ*cspABD*) *csp* mutants were also significantly impaired in cell invasion compared with the wild-type strain. The Δ*cspBD* and Δ*cspABD* mutants displayed the most severely impaired invasion phenotypes. The invasion ability of these two mutant strains was also further analyzed using cold-stress-exposed organisms. In both cell infection models a significant reduction in invasiveness was observed after cold stress exposure of *Listeria* organisms. The negative impact of cold stress on subsequent cell invasion ability was, however, more severe in cold-sensitive *csp* mutants (Δ*cspBD* and Δ*cspABD*) compared with the wild type. The impaired macrophage invasion and intracellular growth of Δ*cspBD* and Δ*cspABD* also led us to examine oxidative stress resistance capacity in these two mutant strains. Both strains also displayed higher oxidative stress sensitivity relative to the wild-type strain. Our data indicate that besides cold and osmotic stress adaptation roles, Csp family proteins also promote efficient host cell invasion and oxidative stress adaptation processes in *L. monocytogenes*.

Introduction

The gram-positive nonsporulating bacterium *Listeria monocytogenes* is responsible for serious public health and food safety problems (Farber and Peterkin, 1991; Ramaswamy *et al.*, 2007; Swaminathan and Gerner-Smidt, 2007). Transmission of these pathogens is mainly foodborne, and these organisms target individuals with weakened immune systems, such as pregnant women, newborns, elderly persons, and those who are immunocompromised. Infections in these groups can lead to listeriosis, a rare disease that causes severe illnesses and high mortality, as well as abortions and still births in pregnant women (Vazquez-Boland *et al.*, 2001; Posfay-Barbe and Wald, 2009). The pathogenic adaptation of this bacterium depends on the execution of a wide range of well-coordinated molecular events designed to resist host defenses and facilitate the infection of different target host cells, which include the intestinal epithelial cells and macrophages. To achieve this, *L. monocytogenes* also possess various molecular virulence mechanisms (see reviews in Vazquez-Boland *et al.*, 2001; Dussurget, 2008). Expression of virulence genes in *L. monocytogenes* is controlled through complex gene expression regulatory networks. These include the central virulence regulating protein PrfA and alternative sigma factor SigB, as well as the two-component-based ViR/VirS virulence regulatory protein system (Mengaud *et al.*, 1991; Mandin *et al.*, 2005; Chaturongakul *et al.*, 2008). The modulation of PrfA regulatory activities based on various environmental cues received by the bacterium seems to be one of the key regulatory events in coordinating the different physiological changes necessary for its transition from free-living saprophytic state to intracellular pathogenic existence (Freitag *et al.*, 2009).

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This process involves a wide range of changes in gene expression based on genome-wide transcriptome analysis of this pathogen, which have to date revealed the activation of various genes during host cell infection processes (Chatterjee *et al.*, 2006; Joseph *et al.*, 2006; Camejo *et al.*, 2009; Toledo-Arana *et al.*, 2009). Although the understanding of key regulatory pathways such as those coordinated through PrfA-based molecular pathways has improved over the years, we still lack extensive knowledge on other regulatory protein systems that may also play a role in modulating gene expression changes that are required to execute various stages of *L. monocytogenes* infection.

Three proteins of the cold shock protein (Csp) family (CspA, CspB, and CspD) found in *L. monocytogenes* may also be crucial in modulating a wide range of gene expression in this bacterium. Proteins of this family can potentially influence expression of various genes based on their nucleic acid chaperone-like activities and ability to regulate both transcription and translation events (Phadtare, 2004; Horn *et al.*, 2007). Some of the major prokaryotic Csps investigated to date have primarily been linked to cold acclimation functions. In addition, it has also been observed that bacterial Csps are involved in execution of other cellular functions such as normal bacterial growth, adaptation to nutrient starvation, and stationary-phase growth (Graumann and Marahiel, 1998). The Csp protein family has been extensively investigated in *Escherichia coli*, where such examples of functional variability have been observed (Yamanaka *et al.*, 1998). Out of the nine Csps (CspA to I), only five (CspA, CspB, CspE, CspG, and CspI) have been associated with cold-adaptation-related functions, whereas others are not involved in cold acclimation (Goldstein *et al.*, 1990; Lee *et al.*, 1994; Nakashima *et al.*, 1996; Wang *et al.*, 1999). Two other Csps (CspC and CspE) of this bacterium are involved in chromosomal condensation and cell division processes as well as in modulating expression of general stress response genes, *rpoS* and *uspA* (Hu *et al.*, 1996; Harrington and Trun, 1997; Bae *et al.*, 1999; Phadtare and Inouye, 2001). The *E. coli* CspD protein has been linked with adaptation to nutritional starvation and stationary growth phase stress (Yamanaka and Inouye, 1997). Moreover, based on studies in other microorganisms, Csp family proteins have also been linked to modulation of microbial adaptive responses to antibiotics, elevated osmolarity, ionic compounds, and oxidative stress conditions (Katzif *et al.*, 2003; Chanda *et al.*, 2009; Schmid *et al.*, 2009). The capacity of Csps to affect multiple gene expression patterns is inferred from the various global gene expression changes induced at transcription and protein levels upon deletion or overexpression of some bacterial *csp* genes (Graumann *et al.*, 1997; Wouters *et al.*, 2000; Phadtare and Inouye, 2001, 2004; Katzif *et al.*, 2005).

The different possible functional roles of the three Csps of *L. monocytogenes* are yet to be understood in more detail. It has been shown that *cspA*, *cspB*, and *cspD* transcripts, as well as Csp-like proteins in this bacterium, are expressed under optimal (37°C) and cold stress (4°C and 10°C) growth temperature conditions in brain heart infusion (BHI) broth cultures (Wemekamp-Kamphuis *et al.*, 2002; Schmid *et al.*, 2009). Strong induction of *cspA* and *cspD* transcripts is observed in response to cold and osmotic stress, but *cspB* is only weakly induced by cold stress (Schmid *et al.*, 2009). The analysis of different *csp* deletion mutants of this bacterium recently showed that *cspA* and *cspD* gene functions facilitate cold growth and osmotic stress tolerance (Schmid *et al.*, 2009). The main functional contributions of *cspB* in this organism are thus not yet clear. We hypothesized that, as observed in other bacterial species, the *L. monocytogenes* Csp protein family might also functionally influence the expression of genes associated with various other physiological functions besides cold and osmotic stress adaptation. We therefore investigated whether Csps in this bacterium might also influence the execution of some host cell infection processes. To this end, a phenotypic evaluation of cellular invasion phenotypes in seven *L. monocytogenes* EGDe *csp* gene family deletion mutants was performed using human Caco-2 and murine macrophage (J744A.1)–based *in vitro* cell infection models.

Materials and Methods

Bacterial strains and cell lines

The *L. monocytogenes* EGDe wild-type strain and various *csp* gene family deletion mutants used in this study are listed in Table 1. The seven *csp* gene family mutants were constructed in-frame by homologous recombination and confirmed by sequencing as previously described (Schmid *et al.*, 2009). These strains were available as frozen stocks at −70°C. They were cultivated on BHI (Oxoid, Basingstoke, Hampshire, United Kingdom) agar plates by incubating at 37°C until single colonies developed (18 hours). The human colorectal epithelial Caco-2 (ATCC-HTB-37) cell line was maintained in minimum essential medium containing 10% fetal calf serum, 1% minimum essential medium non-essential amino acids, 2% GlutaMAX-1, and 0.4% gentamicin (40 mg/mL). The murine monocyte–macrophage J774A.1 (ATCC-TIB-67) cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum. All the cell culture media, additives, and the phosphate-buffered saline (PBS) solution were all sourced from Gibco–Invitrogen AG (Basel, Switzerland).

Quantification of Listeria *organisms by colony counting*

The numbers of *Listeria* organisms in the different inocula and the infected Caco-2 and macrophage cell lysates were quantified by standard colony counting. Bacterial broth cultures or eukaryotic cell lysates were 10-fold serially diluted using 0.85% NaCl–peptone solution. The appropriate dilution series range were plated out on Plate Count-Agar plates

Table 1. Strains Used in This Study

Strain	*Genetic deletion*[a]	*Reference*
EGD-e	None	
Δ*cspA*	*cspA*	Schmid *et al.* (2009)
Δ*cspB*	*cspB*	Schmid *et al.* (2009)
Δ*cspD*	*cspD*	Schmid *et al.* (2009)
Δ*cspAB*	*cspA* and *cspB*	Schmid *et al.* (2009)
Δ*cspAD*	*cspA* and *cspD*	Schmid *et al.* (2009)
Δ*cspBD*	*cspB* and *cspD*	Schmid *et al.* (2009)
Δ*cspABD*	*cspA*, *cspB*, and *cspD*	Schmid *et al.* (2009)

[a]All *csp* gene deletion mutants were created by in-frame deletion of the target gene regions of the *Listeria monocytogenes* EGDe genome as previously described in Schmid *et al.* (2009).

(Oxoid; CM 0325) and incubated at 37°C for 24 hours before the bacterial colonies were counted.

Listeria *inocula*

The first type of *Listeria* inocula used in this study was derived from stationary-phase organisms grown at 37°C. Single colonies from the *L. monocytogenes* strains were grown (12–16 hours) in 10 mL BHI cultures incubated at 37°C in a shaking incubator set to 150 rpm. This process gave rise to stationary-phase *Listeria* cells equivalent to $\sim 10^9$ CFU/mL in each strain, which were subsequently diluted to 10^8 and 10^7 CFU/mL in Iscove's modified Dulbecco's medium (IMDM) and DMEM, respectively. The second type of *Listeria* inocula comprised of stationary-phase cells initially grown as above and subsequently exposed to cold stress. The stationary-phase cultures prepared as described were centrifuged (5000 *g* for 5 minutes) and resuspended in 10 mL of fresh BHI. Such cultures were exposed to cold stress by incubation at 4°C for a further 12 hours. The numbers of *Listeria* in such inocula were determined before and after cold stress exposure to confirm that under these conditions there was no significant growth resumption or bacterial death induced. The cold-stress-exposed *Listeria* were similarly adjusted to 10^8 and 10^7 CFU/mL in IMDM and DMEM.

Caco-2 cell invasion and adhesion assays

Invasion assays with human Caco-2 cells were performed as previously described (Garner *et al.*, 2006). Briefly Caco-2 cells were seeded at 2.0×10^5/mL per well in a 24-well tissue culture plate (TPP AG, Trasadingen, Switzerland) and grown to confluence (37°C under a 5% CO_2 atmosphere) in IMDM. The *Listeria* inocula described above were applied to Caco-2 cell monolayers at ratios of ~250 bacterial organisms per cell. At 45 minutes postinfection, nonadherent bacteria were removed by washing the monolayers three times using sterile PBS. The Caco-2 cell monolayers were overlaid with IMDM plus 10 μg/mL gentamicin and incubated for 1 hour to kill adherent extracellular bacterial organisms. After this step the cell monolayers were once again washed three times with sterile PBS and then lysed by suspension in 1 mL of cold sterile distilled water. The numbers of viable intracellular bacteria were determined by colony counting of appropriate 10-fold serial dilutions of the Caco-2 cell lysates.

The adhesion ability of selected *L. monocytogenes* strains was determined as previously described (Jensen *et al.*, 2008) with some minor modifications. Caco-2 cells were infected as described above, but two sets of duplicated infection experiments per strain were prepared. The nonadherent bacteria were washed off at 45 minutes postinfection with PBS. Thereafter, one set of duplicated samples was overlaid in IMDM plus gentamicin (to kill adherent bacteria), whereas the control set was similarly overlaid but using antibiotic-free IMDM. After 1 hour both sets of samples were washed and processed as above to determine the numbers of viable bacteria. The amount of *Listeria* organisms adhering to Caco-2 cell was subsequently calculated by substraction of intracellular (gentamicin-treated samples) viable bacteria from total cell associated bacterial numbers determined for control samples not exposed to gentamicin. Thereafter, adhesion indexes were determined as numbers of adhered bacteria divided by the amount of the original inoculum and expressed as a percentage.

Macrophage invasion assays

The invasion and intramacrophage growth assays in murine macrophages were performed as previously described (Dubail *et al.*, 2006) with some modifications. The murine-derived J774A.1 macrophages were seeded in at 4.0×10^5 cells per well in a 24-well tissue culture plate and grown (37°C under a 5% CO_2) to confluence in DMEM. The *Listeria* inocula prepared and diluted in DMEM as described above were used to inoculate macrophages at ~10 bacteria per cell. After 60 minutes of infection, the macrophages were washed three times using sterile PBS. The remaining extracellular bacteria were killed by overlaying the macrophage monolayers with DMEM containing 10 μg/mL of gentamicin. The infections were stopped at 1 (invasion assays), 2, 4, and 6 hours (intramacrophage growth assays). The DMEM was removed, and macrophages were washed three times with sterile PBS and similarly processed as described for Caco-2 cells to determine numbers of viable intramacrophage bacteria.

Oxidative stress exposure assays

The oxidative stress survival phenotypes of the EGDe wild-type, Δ*cspBD*, and Δ*cspABD* cells were evaluated in the BHI growth medium containing the oxidative stress agent cumene hydroxyperoxide (CHP) as previously described (Ferreira *et al.*, 2001). The strains were grown to stationary phase by an overnight (12–16 hours) incubation at 37°C in 10 mL BHI as described above. At this stage, 900 μL of culture was mixed with 100 μL dimethyl sulfoxide containing 150 mM CHP, resulting in a final concentration of 15 mM CHP. Aliquots were collected at 0 and 15 minutes of incubation at 37°C, and viable bacterial cell numbers in each sample were determined by colony counting on plate count agar. The number of survivors in each sample after 15 minutes of oxidative stress exposure was expressed as a percentage of original inoculum present at time point zero.

Statistical analysis

The statistical analysis was carried out using the JMP statistical software package (version 7.0; SAS Institute, Cary, NC). The experimental results are all based on the mean of at least three independent assays. The numbers of viable bacteria (CFU/mL) determined by colony counting in cell invasion, adhesion, and oxidative stress experiments were all expressed as percentages of organisms in the corresponding original *L. monocytogenes* inoculum. The statistical significance of differences in cell invasion, adhesion, and oxidative stress survival was evaluated using the Kruskal–Wallis/Wilcoxon's rank-sum tests. For the intramacrophage and BHI-based growth analysis, the bacterial colony counts were normalized by log conversion ($\log_{10}$ CFU/mL) followed by determination of means and standard deviations. The statistical significance of intramacrophage strain growth was evaluated based on pairwise comparison of log-converted intramacrophage bacterial levels after 6 hours ($t = 6$ hours) of infection to those at initial invasion ($t = 0$) using the Student *t*-test. In all cases of statistical analysis differences were considered significant when *p*-values were <0.05.

Results

The cell invasion abilities of ΔcspBD *and* ΔcspABD *strains are severely impaired*

The invasion capacity of the seven *csp* gene family deletion mutants was first investigated based on the inocula of stationary-phase organisms grown at 37°C in the Caco-2 cell infection model (Fig. 1A). Although the Caco-2 cell invasion levels of the Δ*cspA* strain did not significantly ($p > 0.05$; pairwise comparison Wilcoxon rank-sum test) differ from the wild type, those of Δ*cspB* ($p = 0.0054$; pairwise comparison Wilcoxon rank-sum test) and Δ*cspD* ($p = 0.0133$; pairwise comparison Wilcoxon rank-sum test) strains were slightly but significantly lower compared with the wild-type strain. These two single *csp* deletion mutants displayed invasion levels that were three- and twofold, respectively, less than the wild-type strain. Among the double *csp* mutants, both Δ*cspAB* and Δ*cspAD* strains also displayed slight but significantly ($p < 0.05$; overall Kruskal–Wallis rank-sum test) lower invasion than the wild-type strain. The mean percent invasions achieved by Δ*cspAB* and Δ*cspAD* strains were four- and twofold less than the wild-type strain. Meanwhile, Caco-2 invasion levels achieved by the Δ*cspB*, Δ*cspD*, Δ*cspAB*, and Δ*cspAD* strains were not significantly different ($p > 0.05$; overall Kruskal–Wallis rank-sum test). Interestingly, the Δ*cspBD* ($p = 0.0055$; pairwise comparison Wilcoxon rank-sum test) and Δ*cspABD* ($p = 0.0014$; Wilcoxon rank-sum test) strains both displayed Caco-2 cell invasion levels that were even more significantly below those of the wild-type strain and the rest of the *csp* mutants (see Fig. 1A). These two mutants invaded Caco-2 cells at levels that were 19-fold (Δ*cspBD*) and 163-fold (Δ*cspABD*) below those of the wild type.

Next, the invasion capacity of all *csp* mutants was also similarly investigated in a murine macrophage-based cell infection model (see Fig. 1B). Once again, Δ*cspA* invasion levels were similar to the wild-type strain. Although slight reductions were observed in macrophage invasion levels of Δ*cspB*, Δ*cspD*, Δ*cspAB* and Δ*cspAD* strains compared with the parental wild-type strain, such differences were, however, found to be not statistically significant in this infection model, but similar to observations in Caco-2 cells, the macrophage invasion levels of Δ*cspAB* and Δ*cspAD* strains were once again significantly ($p < 0.05$; overall Kruskal–Wallis rank-sum test) below those of the wild-type strain in this cell infection model. The mean macrophage invasion levels of Δ*cspBD* were fivefold, whereas those of Δ*cspABD* were 10-fold lower than the wild-type strain.

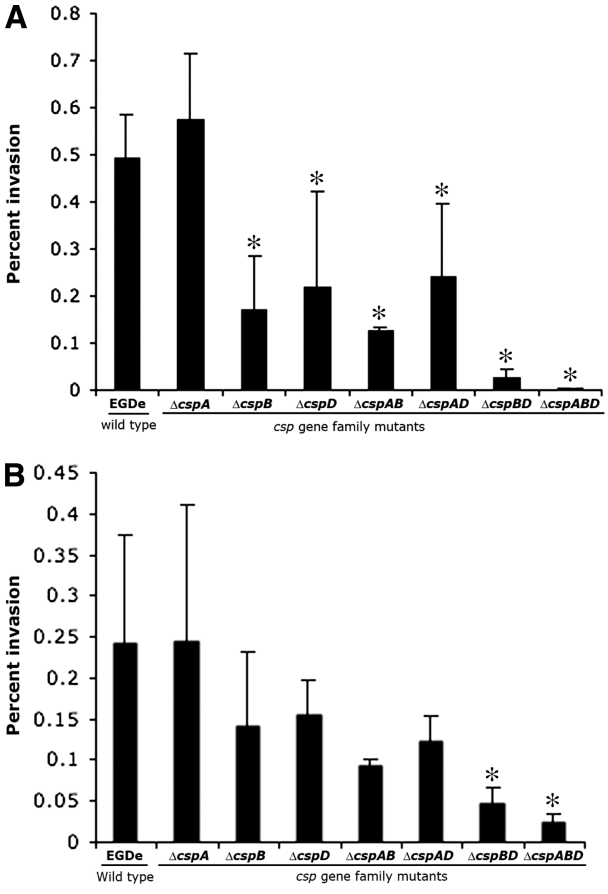

FIG. 1. Caco-2 (**A**) and J774A.1 macrophage (**B**) cell percent invasion of *Listeria monocytogenes* EGDe wild-type and seven *csp* deletion mutant derivatives based on the inocula of stationary-phase organisms grown at 37°C. The percentage invasions (percentage of original bacterial inoculum recovered from the infected Caco-2 or J774A.1 macrophage cells) are displayed on the y-axis. The eight strains were evaluated in duplicates per experiment, and bars reflecting the mean and error (±1 SD) based on three independent assays for each strain are presented. All *csp* deletion mutants displaying significantly reduced host cell invasiveness ($p < 0.05$; Wilcoxon/Kruskal–Wallis rank-sum test) compared with the wild-type strain are denoted by an asterisk above the bars. SD, standard deviation.

Cold stress exposure significantly impairs L. monocytogenes *host cell invasion*

Differences in invasion ability of wild-type, Δ*cspBD*, and Δ*cspABD* strains were further examined using bacteria initially grown until stationary phase at 37°C and subsequently cold adapted by 12 hours of incubation at 4°C (see Fig. 2A, B). This analysis revealed that cold stress exposure generally impacted negatively on the subsequent host cell invasion ability of three analyzed *L. monocytogenes* strains. The host cell invasions of cold-stress-exposed wild-type as well as Δ*cspBD* and Δ*cspABD* mutant strains were all significantly below those of their corresponding non-cold-stress-exposed inocula ($p < 0.05$; overall Kruskal–Wallis rank-sum test). However, the extent to which host cell invasion decreased postcold stress exposure differed depending on the strain as well as infected host cell type. In Caco-2 cells we found that the percent invasions of wild-type, Δ*cspBD*, and Δ*cspABD* strains were 5-, 262-, and 58-fold reduced after cold stress exposure. In macrophages the invasion of cold-stress-exposed wild type was only ninefold reduced after cold stress exposure, whereas that of Δ*cspBD* and Δ*cspABD* was 441- and 1141-fold, respectively, reduced. The more important observation was, however, that this invasion analysis using cold-stress-exposed inocula of these three strains further confirmed that Δ*cspBD* and Δ*cspABD* mutants are significantly impaired in host cell invasion relative to the wild-type strain. The Δ*cspBD* and Δ*cspABD* strains demonstrated Caco-2 cell invasions that were on average 350- and 875-fold below the wild type,

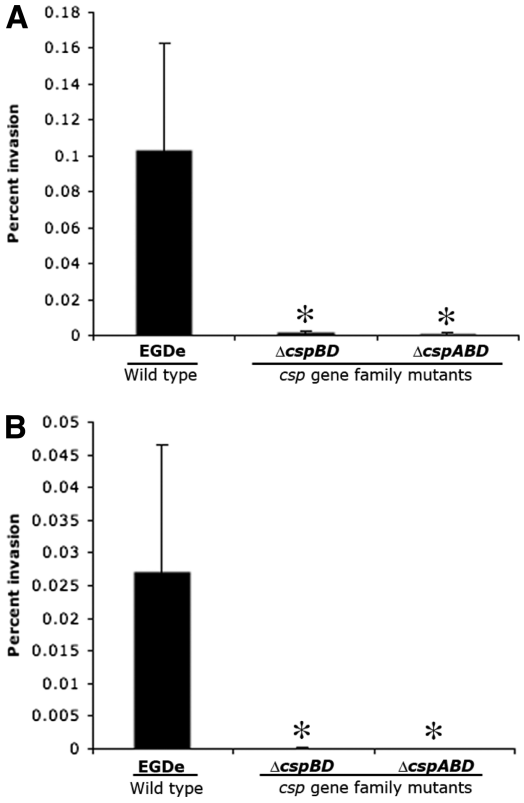

FIG. 2. Caco-2 (**A**) and J774A.1 macrophage (**B**) cell percent invasion of wild-type, Δ*cspBD*, and Δ*cspABD* strains based on inocula of cold-stress-exposed organisms. The percent invasions (percentage of original bacterial inoculum recovered from the infected Caco-2 or J774A.1 murine macrophage cells) are displayed on the y-axis. The three strains were evaluated in duplicates per experiment, and bars reflecting the mean and error (±1 SD) based on three independent analyses of each strain are presented. The Δ*cspBD* and Δ*cspABD* strains displayed significantly ($p < 0.05$; Wilcoxon/Kruskal–Wallis rank-sum test) reduced host cell invasion compared with the wild-type strain as denoted by an asterisk above the bars.

whereas their macrophage invasiveness was 441- and 1213-fold, respectively, lower than wild type.

The ΔcspBD *and* ΔcspABD *strains are not significantly impaired in adherence to the Caco-2 cell surfaces*

Cell surface adhesion is a crucial step during Caco-2 cell invasion, and thus we also examined the adhesion abilities of wild-type, Δ*cspBD*, and Δ*cspABD* strains in this cell infection model (see Fig. 3). The Δ*cspBD* and Δ*cspABD* strains adhered to Caco-2 cell surfaces with mean adhesion indexes of $28.3 \pm 3.9\%$ and $27.9 \pm 8.3\%$, respectively. The mean adhesion indexes displayed by these two *csp* mutants in this cell infection model were significantly higher ($p < 0.05$; overall Kruskal–Wallis rank-sum test) than their parental wild-type strain, which displayed a $16 \pm 3.4\%$ mean adhesion index under these conditions. This indicates that poor Caco-2 cell invasion phenotypes displayed in these two *csp* mutants are not linked to a reduced ability of these organisms to attach to Caco-2 cell surfaces.

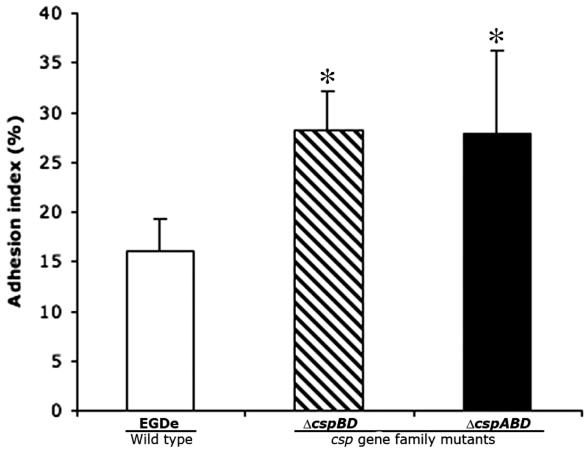

FIG. 3. Caco-2 cell adhesion indexes of wild-type, Δ*cspBD*, and Δ*cspABD* strains based on inocula derived from stationary-phase organisms grown at 37°C. The adhesion indexes (Caco-2 cell-surface-adherent organisms expressed as a percentage of original bacterial inoculum) of the strains are displayed on the y-axis. The three strains were evaluated as duplicates per experiment, and bars reflecting the mean and error (±1 SD) based on three independent analyses of each strain are presented. Statistically significant differences ($p < 0.05$; Wilcoxon/Kruskal–Wallis rank-sum test) in the adhesion indexes of Δ*cspBD* and Δ*cspABD* strains compared with the wild-type strain are denoted by an asterisk above the bars.

The ΔcspABD *mutant displays impaired intramacrophage growth ability*

The growth properties of wild-type, Δ*cspBD*, and Δ*cspABD* strains were investigated in BHI cultures as well as infected macrophages during incubation at 37°C (Fig. 4A, B). There were no significant differences in growth of the three strains in BHI cultures, but some variability in intramacrophage growth was observed. Similar intramacrophage growth rates were determined for wild-type and Δ*cspBD* strains (data not shown). The Δ*cspABD* strain on the other hand did not display significant growth in infected macrophages. Both wild-type ($p = 0.0004$; pairwise comparison Student *t*-test) and Δ*cspBD* ($p = 0.0071$; pairwise comparison Student *t*-test) intramacrophage levels were significantly higher after 6 hours of infection than those present at initial invasion. In contrast, intramacrophage Δ*cspABD* levels at 6 hours after infection were not significantly higher ($p > 0.05$; pairwise comparison Student *t*-test) than those present at initial invasion. These results thus indicate that although all three strains grow similarly under BHI nutrient conditions, intracellular growth of Δ*cspABD* is impaired in infected macrophages, whereas that of Δ*cspBD* mutant does not differ from the wild-type strain.

The ΔcspBD *and* ΔcspABD *strains are impaired in oxidative stress tolerance*

The survival capacity of wild-type, Δ*cspBD*, and Δ*cspABD* strains exposed to oxidative stress in the BHI medium supplemented with 15 mM CHP was also examined (see Fig. 5). As shown the Δ*cspBD* and Δ*cspABD* mutants were more sensitive to oxidative stress than the wild-type strain. After

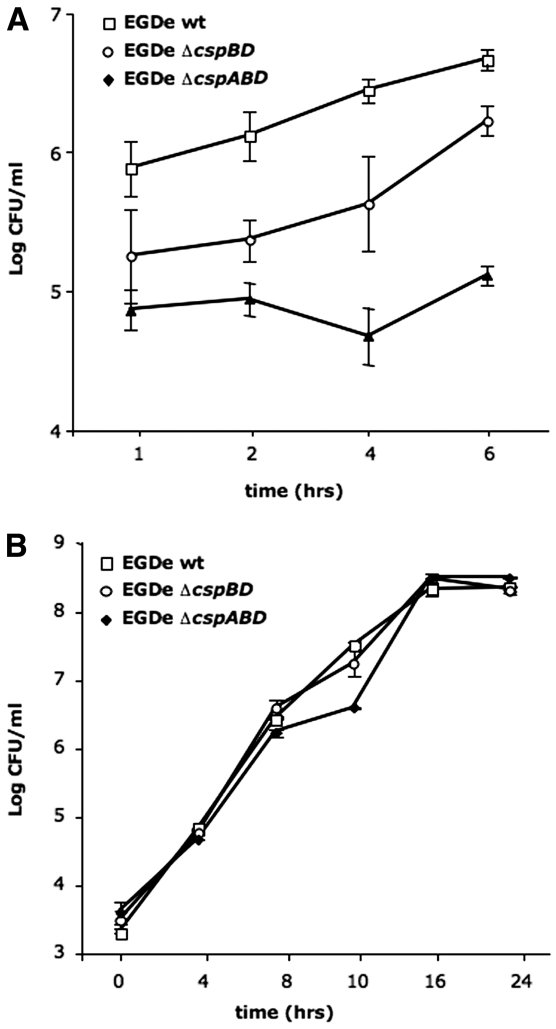

FIG. 4. Growth behavior of wild-type (□), Δ*cspBD* (○), and Δ*cspABD* (▲) strains in (**A**) infected J774.1 macrophages cultured at 37°C and (**B**) brain heart infusion culture in a shaking incubator held at 37°C. The three strains were analyzed as duplicates per experiment in three independent assays, and bars reflecting the mean and error (±1 SD) based on three independent analyses of each strain are presented.

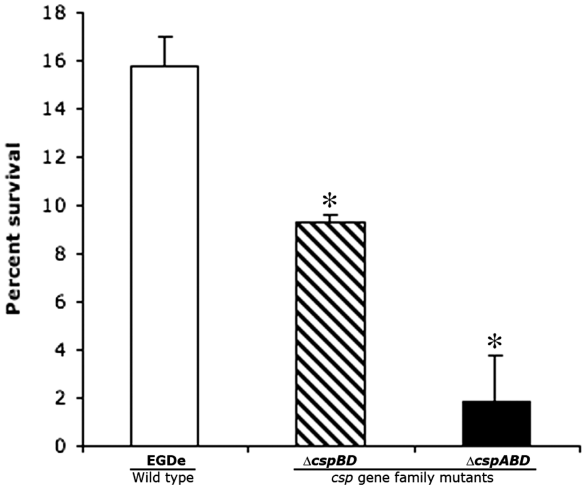

FIG. 5. Oxidative stress survival capacity of wild-type, Δ*cspBD*, and Δ*cspABD* strains. The percent survival (percentage of original inoculum surviving after 15 minutes in brain heart infusion plus 15 mM cumene hydroxyperoxide at 37°C) of the strains is displayed on the y-axis. The three strains were analyzed as duplicates in three independent assays, and bars reflecting the mean and error (±1 SD) based on three independent analyses of each strain are presented. The Δ*cspBD* and Δ*cspABD* strains displayed significantly ($p < 0.05$; Wilcoxon/Kruskal–Wallis rank-sum test) lower survival of oxidative stress conditions compared with the wild-type strains as indicated by an asterisk above each bar.

15 minutes of oxidative stress exposure, the Δ*cspBD* and Δ*cspABD* mutants had 9.3 ± 0.3% and 1.9 ± 1.8% of survivors, respectively. This was significantly ($p < 0.05$; overall Kruskal–Wallis rank-sum test) reduced compared with the wild-type strain, which displayed 15.8 ± 1.3% survival rate under similar conditions.

Discussion

The present study has examined the functional contributions of Csp family proteins in host cell infection processes as well as oxidative stress resistance capacity in *L. monocytogenes*. Based on previous work from our group and others, it has been shown that *csp* gene transcripts as well as Csp-like proteins are detectable in *L. monocytogenes* cells grown under optimal conditions in BHI at 37°C (Wemekamp-Kamphuis *et al.*, 2002; Schmid *et al.*, 2009). Meanwhile, some, but not all, of the *csp* gene transcripts and Csp-like proteins in this organism are activated in response to cold stress conditions (Wemekamp-Kamphuis *et al.*, 2002; Schmid *et al.*, 2009). These observations thus indicate that Csps may be involved in promotion of cellular functions under cold stress as well as during optimal temperature growth conditions in this bacterium. On the basis of this, we thus also hypothesized that Csps might have an influence on physiological functions that may be crucial in host cell infection processes in *L. monocytogenes*. This was more so based on the fact that both *csp* transcripts and Csp-like proteins are expressed at 37°C in this bacterium, suggesting that proteins of this family are also functional at temperatures where human host cell infection processes might probably occur.

We performed a phenotypic analysis of several *csp* gene family-based deletion mutants in comparison to a parental wild-type strain of *L. monocytogenes* EGDe. We applied both human intestinal epithelial (Caco-2) and macrophage-like (murine J744A.1 macrophages) cell-based infection models. These two models were selected to assess the ability of *csp* mutants to invade both nonphagocytic and phagocytic cells based on different cell entry and intracellular adaptive strategies. The invasion phenotypes displayed in these two cell infection models clearly indicate that *csp* functions, particularly those of *cspB* and *cspD* genes, promote efficient *L. monocytogenes* invasion in these two types of host cells. The deletion of *cspB* and *cspD* functions in the context of single *csp* gene mutants shows slight but statistically significant reductions in Caco-2 cell invasion capacity relative to wild-type strain. Although macrophage invasion levels displayed of these two single *csp* mutants are also less compared to the wild-type strain, such invasion phenotypic differences were

not statistically significant in this infection model. An observation, which might also have been influenced by the different modes of cellular invasion, associated within the two models, since epithelial cell invasion is internalin mediated, whereas macrophage invasion is largely an active uptake process through phagocytosis. In contrast, we found in both cell infection models that deletion of the *cspA* gene alone does not significantly affect *L. monocytogenes* EGDe invasion while both *cspB* and *cspD* functions are intact. The combined loss of *cspB* and *cspD* in Δ*cspBD* or loss of all three *csps* (*cspA*, *cspB*, and *cspD*) genes in Δ*cspABD* leads to more severe impairment of host cell invasion in *L. monocytogenes* EGDe. These two *csp* deletion mutants showed significantly lower invasion compared with the wild-type strain, as well as in comparison to the five (Δ*cspA*, Δ*cspB*, Δ*cspD*, Δ*cspAB*, and Δ*cspAD*) other *csp* deletion mutants. The Δ*cspBD* and Δ*cspABD* invasion phenotype defects were confirmed based on organisms grown at 37°C and those preadapted to cold stress conditions. Although findings in these mutants in future need further confirmation based on genetic complementation, our results suggest that in *L. monocytogenes* as long as either *cspB* or *cspD* genes remain intact, loss of other *csp* genes has limited impact on cell invasion processes in this bacterium. Besides indicating functional contributions of these two *csp* genes in *L. monocytogenes* cell invasion, observations in this work also suggest functional redundancy between these two *csps* in context of their roles in this process. Molecular function redundancy between *cspB* and *cspD* might be expected given the high levels of nucleotide (74%) and amino acid (67%) sequence identity between these two *csp* genes in *L. monocytogenes* (Schmid *et al.*, 2009).

Previous studies have also shown that *cspA* is the main *csp* gene contributing to efficient cold adaptation and low temperature growth in this bacterium (Schmid *et al.*, 2009). Deletion of this gene abolishes growth of this bacterium under cold stress both at 4°C and 10°C. The contribution of this Csp to host cell invasion processes, however, seems less significant compared with CspB and CspD based on our findings in the current study. As long as one or both of these two *csps* remain intact, it seems that loss of *cspA* function has no consequences on cell invasion phenotypes displayed by *L. monocytogenes* as no significant cell invasion defects were detected for the Δ*cspA* strain in both cell infection models. Similarly, phenotypic invasion defects induced by *cspB* and *cspD* deletion do not seem to be further exacerbated by *cspA* deletion in most cases as shown in the context of Δ*cspAB* and Δ*cspAD* double *csp* deletion mutants. The Caco-2 and macrophage cell invasions displayed between Δ*cspB* and Δ*cspAB*, as well as those between Δ*cspD* and Δ*cspAD* strain pairs, were not significantly different ($p > 0.05$; Wilcoxon/Kruskal–Wallis rank-sum test). The functional contribution of *cspA* to some aspects of host cell invasion phenotypes, however, also becomes apparent in backgrounds where both *cspB* and *cspD* gene functions are missing as shown in context of the double (Δ*cspBD*) and triple (Δ*cspABD*) *csp* deletions. This, for example, was observed in Caco-2 cell invasion analysis using *L. monocytogenes* inocula grown at 37°C. In this case, the Δ*cspABD* mutant strain showed 11-fold lower invasiveness compared with a Δ*cspBD* mutant. Further, we also found that while intramacrophage growth was intact in a Δ*cspBD* strain, there was Δ*cspABD* growth failure within infected macrophages. In both these cases the phenotypic differences seem to be linked to intact *cspA* gene functions in Δ*cspBD* strain, which are missing in the Δ*cspABD* mutant strain background.

In the murine macrophages strongly reduced invasion capacities of Δ*cspBD* and Δ*cspABD* mutants relative to the wild-type strain were also observed, whereas intramacrophage growth analysis further revealed impaired intracellular growth ability in the Δ*cspABD* compared with the wild-type or Δ*cspBD* strains. Intracellular environmental stress encountered by *L. monocytogenes* in macrophages includes oxidative stress conditions. It has also been recently shown that expression of some bacterial *csps*, such as the *cspC* gene of *Staphylococcus aureus*, is in fact more strongly activated in response to oxidative than cold stress exposure (Chanda *et al.*, 2009). Such findings are thus suggestive of some Csp-associated functional roles in bacterial oxidative stress adaptation. We therefore wondered if the *csp* family genes missing in *L. monocytogenes* Δ*cspBD* and Δ*cspABD* strains could also be contributing to oxidative stress tolerance in this bacterium. Defective oxidative stress tolerance could in part explain the poor macrophage invasion in both *csp* deletion mutants, as well as intramacrophage growth failure of the Δ*cspABD* mutant. Interestingly, these two *csp* mutants were both also found to be more sensitive to oxidative stress exposure than the wild-type strain. Moreover, the Δ*cspABD* mutant was significantly more sensitive of oxidative stress ($1.9 \pm 1.8\%$ vs. $9.3 \pm 0.3\%$ survival) than the Δ*cspBD* strain ($p = 0.0098$; Wilcoxon/Kruskal–Wallis rank-sum test). Since the Δ*cspBD* mutant still has an intact *cspA* gene and survives oxidative stress better than a Δ*cspABD* mutant, these findings also further suggest direct *cspA* functional contributions in *L. monocytogenes* oxidative stress tolerance. Meanwhile, based on these observations we also conclude that the poor macrophage invasion phenotypes of Δ*cspBD* and Δ*cspABD* mutants, as well as intramacrophage growth failure in Δ*cspABD*, may partly be due to their reduced ability to survive when exposed to vacuolar oxidative stress environments.

Previous studies based on a chick embryo virulence model have described reduced pathogenicity of meat-processing-plant-derived *L. monocytogenes* strains induced by cold stress exposure (Buncic and Avery, 1996). Similarly, in the present study we demonstrated that cold stress exposure reduces host cell invasion ability of the EGDe wild-type strain as well as that of the Δ*cspBD* and Δ*cspABD* strains. In case of the wild-type strain, the percent invasion displayed in Caco-2 and macrophages was fivefold and ninefold, respectively, reduced postcold stress exposure. On the other hand, the extent of cold stress exposure on the subsequent cell invasion capacity of the two *csp* mutant strains varied depending on the infected host cell type. In Caco-2 cells we observed that exposure to cold stress reduced the invasion of Δ*cspBD* and Δ*cspABD* by 262- and 58-fold, respectively. The macrophage invasion of these two strains on the other hand was reduced 441- and 1213-fold, respectively, in cold-stress-exposed organisms. The reasons for the variable effect of *cspA* deletion on invasion ability of cold-exposed organisms in the context of these two multiple *csp* deletion mutants are currently not clear. It may be speculated that cold stress exposure impairs as of yet unknown *cspA*-dependent molecular processes that promote macrophage invasion, but such functions are dispensable in Caco-2 cell invasion mechanisms of this bacterium.

On the basis of the analysis of cold-stress-exposed infecting organisms, we have also demonstrated that the cell invasion capacity of both Δ*cspBD* and Δ*cspABD* mutants is even more

significantly impaired compared with the wild-type strain after cold stress exposure. While the mean Caco-2 cell invasion achieved by Δ*cspBD* and Δ*cspABD* strains were 19- and 163-fold less than wild-type in non-cold-stress-exposed organism, the invasion of these two mutants was 350- and 875-fold, respectively, below those of wild type after cold stress exposure. Similarly, non-cold-stress-exposed Δ*cspBD* and Δ*cspABD* organisms invaded macrophages at levels that were 5- and 10-fold lower than those of wild type, but when cold-stress-exposed inocula were used, the mean invasion levels of these two *csp* mutants were 441- and 1213-fold, respectively, below those of wild-type organisms. On the basis of these observations, we can thus also conclude that *L. monocytogenes* organisms with poor cold stress tolerance become even more severely impaired in host cell invasion ability after exposure to reduced temperature conditions. The reasons for such cold-stress-induced reduction in host cell invasion ability of *L. monocytogenes* are not yet clear, but they may be linked to membrane damage as well as surface protein changes that result from cold stress exposure.

In conclusion, the data provided in this study indicate that some *csp*-gene-family-encoded functions promote efficient host cell invasion and oxidative stress tolerance in *L. monocytogenes*. The exact molecular events associated with *csp* functional roles in these phenotypes are, however, not yet understood. Csps are known to influence various microbial gene expression patterns in other bacterial organisms. Based on this, it can be hypothesized that some of Csp-dependent gene expression modulations involve expression of proteins with roles in host cell infection and oxidative stress adaptation processes of *L. monocytogenes*. This study has provided evidence in support of *csp* roles in these processes and thus justifies future investigations to define *csp*-influenced gene expression and, more importantly, the identification of potential virulence-promoting processes that are modulated through Csp-dependent mechanisms in this bacterium.

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Disclosure Statement

No competing financial interests exist.

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